GD2 LIGANDS

FIELD OF THE INVENTION

The invention includes therapeutic compositions and methods, such as peptide cancer chemotherapeutic agents.

BACKGROUND OF THE INVENTION

Gangliosides are cell surface glycosphingolipids containing one or more sialic acid residues. It has been suggested that gangliosides may be localized within detergent-resistant cell membrane microdomains termed "rafts", which may provide the environment for some proteins to function by bringing together adapter molecules, modifiers, substrates, or cofactors that would be otherwise too distant or too dilute to form complexes and activate a signal cascade. However, little has been proposed regarding the possible mechanism of action of gangliosides in signal transduction.

Ganglioside GD2 is reportedly expressed at low levels in certain neuronal populations, but is highly prevalent in many types of tumors (neuroblastoma, melanoma, small cell carcinoma of the lung, gliomas, soft tissue sarcomas and B cell lymphoma).

The extracellular matrix component Tenascin-R was described recently as a natural ligand for GD2 (Probstmeier et al., 1999). However, relatively little is known about the biological function(s) of GD2 and the functional nature of its interaction with ligands such as Tenascin-R.

GD2 has been extensively studied as a tumor marker and is used clinically as a target for antibody-mediated therapy (e.g. anti-GD2 mAb 3F8) (Cheung et al., 1985). However, anti-GD2 mAb 3F8 applied therapeutically to patients causes acute and transient pain immediately after administration. Anti-GD2 mAb 3F8-based therapeutics have been suggested for use in a wide range of cancer

therapeutics and diagnostics, including neuroblastoma and leptomeningeal cancer. For example, ^{131}I-labeled anti-GD2 3F8 monoclonal antibody has been used in targeted radioimmunotherapy (dosed at 20 mCi/kg) in conjunction with immunotherapy with 400 mg/m2 unlabeled/unmodified 3F8. Similarly, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been used in conjunction with anti-GD2 monoclonal antibody 3F8 in the treatment of patients with neuroblastoma.

SUMMARY OF THE INVENTION

In one aspect, the invention provides GD2 ligands of Formula I:

$$Z_1 - X_1 - X_2 - X_3 - X_4 - X_5 - X_6 - X_7 - X_8 - X_9 - X_{10} - X_{11} - X_{12} - X_{13} - Z_2 \quad (I)$$

wherein

X_1 is absent or Y or an analogue thereof;

X_2 is absent or C or an analogue thereof;

X_3 is G or Y or an analogue thereof;

X_4 is G or C or Y or an analogue thereof;

X_5 is I or C or an analogue thereof;

X_6 is T or A or an analogue thereof;

X_7 is N or an analogue thereof;

X_8 is Y or an analogue thereof;

X_9 is N or G or an analogue thereof;

X_{10} is S or C or V or T or an analogue thereof;

X_{11} is A or C or Y or H or S or an analogue thereof;

X_{12} is absent or L or C or Y or an analogue thereof;

X_{13} is absent or M or Y or an analogue thereof;

Z_1 is an N-terminal group of the formula H2N-, RHN- or, RRN-;

Z_2 is a C-terminal group of the formula -C(O)OH, -C(O)R, -C(O)OR, -C(O)NHR, -C(O)NRR;

R at each occurrence is independently selected from (C_1-C_6) alkyl, (C_1-C_6) alkenyl, (C_1-C_6) alkynyl, substituted (C_1-C_6) alkyl, substituted (C_1-C_6) alkenyl, or substituted (C_1-C_6) alkynyl;

and wherein "-" is a covalent linkage.

In alternative embodiments, the invention provides substantially pure synthetic GD2 ligands or recombinant GD2 ligands having a domain of Formula II:

$$- X_1 - X_2 - X_3 - X_4 - X_5 - X_6 - X_7 - X_8 - X_9 - X_{10} - X_{11} - X_{12} - X_{13} - \text{(II)}$$

wherein
X_1 is absent or Y or an analogue thereof;
X_2 is absent or C or an analogue thereof;
X_3 is G or Y or an analogue thereof;
X_4 is G or C or Y or an analogue thereof;
X_5 is I or C or an analogue thereof;
X_6 is T or A or an analogue thereof;
X_7 is N or an analogue thereof;
X_8 is Y or an analogue thereof;
X_9 is N or G or an analogue thereof;
X_{10} is S or C or V or T or an analogue thereof;
X_{11} is A or C or Y or H or S or an analogue thereof;
X_{12} is absent or L or C or Y or an analogue thereof;
X_{13} is absent or M or Y or an analogue thereof;
and wherein "-" is a covalent linkage.

In one aspect, the invention provides recombinant proteins having domains of the invention, wherein the domain is capable of mediating binding of the recombinant protein to GD2. For example, recombinant T-cell receptors having the domains of the invention may be provided in transformed T-cell lines, such as cytotoxic T-cells (a "cytotoxic T lymphocyte" or "CTL" is an immune system cell that recognises epitopes presented by class I MHC molecules through its TCR.). Transformed T-cell lines of the invention may for example be used to treat diseases such as cancers having pathological tissues characterized by expression of GD2 (similar to an approach described in United States Patent Application 20020018783

A1, published in the name of Sadelain, M. *et al.* on February 14, 2002, incorporated herein by reference).

The GD2 ligands of the invention may further comprise a cyclic linkage between any two of X_1 through X_{13}. In alternative embodiments, the GD2 ligands of the invention, or the domains of the invention, may be selected from the group consisting of: GGITNYNSALM; YCGGITNYNSACY; YCITNYNSCY; YCGGITNYNCY; YCTNYGVHCY; YCTNYGVCY; GGIANYNTS; YCGGIANYNCY; YCGGIANYNTSCY; and, YCIANYNTCY. In some embodiments, known GD2 ligands such as mAb 3F8 and tenascin-R are specifically excluded from the genus of claimed ligands in the present invention. However, in some embodiments, small molecule derivatives and analogs of known ligands (such as small peptides or peptidomimetics of the complementarity determining region of mAbs) are not excluded.

The invention also provides methods of treating a subject having a disease wherein disease cells express GD2, the method comprising administering to the subject an effective amount of the GD2 ligands of the invention. Also provided are methods of diagnosis of a disease wherein disease cells express GD2, comprising determining whether a cell from a subject binds to a GD2 ligand of the invention. The diagnostic and therapeutic methods of the invention may be carried out *in vitro* or *in vivo*.

In alternative embodiments, the GD2 ligands of the invention may be used with other therapeutic compounds, such as an effective amount of granulocyte-macrophage colony-stimulating factor. The invention provides commercial packages comprising the GD2 ligands of the invention, together with instructions for using the GD2 ligands to modulate GD2 activity or to detect cells expressing GD2.

Table 1 sets out the sequences of a number of alternative GD2 ligands or GD2 binding domains of the invention. Structure activity relationship (SAR) and

deletion analysis demonstrate that some substitutions are allowed and that peptide cyclization enhances activity (peptides marked by * are inactive)

Table 1: Alternative domains/peptides of the invention

		G	G	I	T	N	Y	N	S	A	L	M	
Y	C	G	G	I	T	N	Y	N	S	A	C	Y	
		Y	C	I	T	N	Y	N	S	C	Y		
Y	C	G	G	I	T	N	Y	N	C	Y			
			Y	C	T	N	Y	G	V	H	C	Y	*
			Y	C	T	N	Y	G	V	C	Y		*
		G	G	I	A	N	Y	N	T	S			*
Y	C	G	G	I	A	N	Y	N	C	Y			
Y	C	G	G	I	A	N	Y	N	T	S	C	Y	
		Y	C	I	A	N	Y	N	T	C	Y		
X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	

The invention further provides commercial packages comprising a GD2 ligand, together with instructions for using the GD2 ligand, for example by administering the GD2 ligand to a patient in need of such treatment.

In alternative embodiments, the invention provides methods of screening to identify a GD2 ligand. For example, such screening methods may involve:

a) Administering a putative GD2 ligand (such as a polypeptide derived from Tenascin-R) to a system having a GD2 moiety and a $p56^{Lck}$ moiety available for association (such as a cell expressing GD2 and $p56^{Lck}$); and,

b) Measuring an association between the GD2 and the $p56^{Lck}$ moieties in the system, for example by determining a kinase activity of the $p56^{Lck}$ moiety. In further alternative embodiments, the invention may include:

c) Measuring an association between the GD2 and the CD45 moieties in the system, for example by determining the phosphatase activity of the CD45 moiety.

In further alternative embodiments, the forgoing method further comprises:

b) Administering a putative GD2 ligand to a system having a GD2 moiety and a phosphatase such as CD45; and

c) Measuring an association or functional relationship between GD2 and the phosphatase

d) A competitive screening assay where test compounds (e.g. a combinatorial library) are used to displace any of the GD2 ligands described herein

e) A competitive screening assay where any of the GD2 ligands described herein are used to displace test compounds

In some embodiments, the putative GD2 ligand may for example comprise a polypeptide or a non-peptidic analog such as a peptidomimetic that displays the same pharmacophore or has similar side chain functional groups.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the full-length protein sequence of Homo sapiens tenascin-R (restrictin, janusin), from GenBank Accession XP_040550, with a region identified in bold within a box that is identified in accordance with one aspect of the invention as a GD2 binding region. One aspect of the invention provides alternative tenascin-R proteins in which the GD2 binding region has been altered, for example to modulate the strength of the interaction between GD2 and the tenascin-R protein.

Figure 2 shows data relating to the co-immunoprecipitation of GD2 and $p56^{Lck}$:

(a) R1.1 and EL4 $GD2^{positive}$ cell lysates were subjected to immunoprecipitation with anti-GD2 mAb 3F8 or control mouse IgG (mIg). Presence of $p56^{Lck}$ protein in the immunoprecipitates was evaluated by western blotting. $p56^{Lck}$ can be immunoprecipitated by mAb 3F8 in EL4 $GD2^{positive}$ cells (lane 7) but not in R1.1 cells (lane 5). Total cell lysates were used as a control for $p56^{Lck}$ detection (lane 1 and 2).

(b) and (c) R1.1, EL4 GD2^{negative} and EL4 GD2^{positive} cell lysates were subjected to immunoprecipitation with anti-GD2 mAb 3F8, GM1-binding cholera toxin B, anti-p56^{Lck} mAb 3A5 and anti-Zap-70 mAb LR. Presence of p56^{Lck} kinase activity in the immunoprecipitates was evaluated by *in vitro* kinase assay with the p56^{Lck}-specific substrates Sam68 and Gap p62. The data in Figure 2 (b) shows that p56^{Lck} kinase activity can be immunoprecipitated by mAb 3F8 in EL4 GD2^{positive} cells (lane 1) but not in EL4 GD2^{negative} cells (lane 4). The data in Figure 2 (c) shows that p56^{Lck} kinase activity can be immunoprecipitated by mAb 3F8 in EL4 GD2^{positive} cells (lane 1) but by cholera toxin B in R1.1 cells (lane 4).

(d) R1.1 and EL4 GD2^{positive} cell lysates were subjected to immunoprecipitation with anti-GD2 mAb 3F8, GM1-binding cholera toxin B and anti-p56^{Lck} mAb 3A5. Presence of gangliosides GD2 and GM1 in the immunoprecipitates was evaluated by ELISA. Anti-p56^{Lck} mAb 3A5 can immunoprecipitate GD2 from EL4 GD2^{positive} cells, but can not immunoprecipitate GM1 from R1.1 cells. Shown are averages ± SEM of one representative experiment. n=4 per experiment.

Figure 3 shows the effect of gangliosides on the *in vitro* kinase activity of purified p56^{Lck}. The kinase activity of p56^{Lck} was evaluated by measuring the incorporation of $^{32}PO_4$ in a peptidic p56^{Lck} substrate over time, with or without GD2 or GM1. Presence of GD2, but not GM1, can positively alter the kinetics of p56^{Lck} kinase activity. Activity standardized to untreated p56^{Lck} at the 20 minute time point. Shown are averages of 3 to 5 assays ± SEM.

Figure 4 shows:

(a) the effect of GD2 ligands on p56^{Lck} phosphorylation. Resting EL4 GD2^{positive} cells were treated with mAb 3F8 (13 nM) or control mouse IgG (mIg) for the indicated times. After lysis, p56^{Lck} protein was immunoprecipitated with anti-p56^{Lck} mAb 3A5 and probed for phosphotyrosine (PY) by western blotting using biotinylated anti-phosphotyrosine mAb 4G10. Anti-GD2 mAb 3F8 can induce tyrosine phosphorylation of p56^{Lck} within 5 minutes (lane 3) and sustain it for at least 30 minutes (lane 4).

(b) the effect of GD2 ligands on Zap-70 phosphorylation. Resting EL4 $GD2^{positive}$ cells were treated with mAb 3F8 (13 nM) or control mouse IgG (mIg) for the indicated times. After lysis, Zap-70 protein was immunoprecipitated with anti-Zap-70 mAb LR and probed for phosphotyrosine (PY) by western blotting using anti-phosphotyrosine mAb 4G10. Anti-GD2 mAb 3F8 can induce tyrosine phosphorylation of Zap-70 within 5 minutes (lane 3). c) Effect of GD2 ligands on intracellular calcium concentrations. Resting EL4 $GD2^{positive}$ cells were treated with mAb 3F8 (13 nM), control mouse IgG (mIg) or the calcium ionophore A23187. Intracellular calcium concentration was evaluated over time by flow cytometry using the calcium-sensitive fluorophore Rhod-2 AM. mAb 3F8 can induce strong, sustainable calcium changes within 5 minutes, while control mouse IgG has no effect. Addition of the $p56^{Lck}$ inhibitor PP1 partially abolished mAb 3F8's effects. Shown are averages of 4 independent assays ± SEM.

Figure 5 shows the inhibitory effect of GD2 on *in vitro* CD45 phosphatase activity , particularly when using the $pp60^{Src}$ C-terminal phosphoregulatory sequence as a substrate. When co-incubated for 20 minutes, GD2 (but not other gangiosides) can drastically inhibit CD45 phosphatase activity, as measured by the colorimetric quantification of released free phosphate.

Figure 6 shows the inhibitory effect of GD2 on *ex vivo* CD45 phosphatase activity, measured by the dephosphorylation of tyrosine 505 on $p56^{Lck}$. EL4 $GD2^{positive}$ and EL4 $GD2^{negative}$ cells were treated with control mIg, anti-GD2 antibody 3F8 or anti-CD3 antibody 145-2C11 cross-linked with anti-hamster antibody G94-56 for 20 minutes at 37°C. After lysis, $p56^{Lck}$ was immunoprecipitated with anti-$p56^{Lck}$ 3A5 coated beads and probed for phosphotyrosine at position 505 by western blotting with anti-PY505 antibody. EL4 $GD2^{positive}$ cells treated with anti-CD3 fail to induce dephosphorylation of $p56^{Lck}$ at tyrosine 505 (lane 3), while EL4 $GD2^{negative}$ cells show a marked dephosphorylation with the same treatment (lane 6), indicating that the presence of GD2 inhibits the CD45 phosphatase responsible for tyrosine 505 dephosphorylation. GD2 ligands such as anti-GD2 antibody 3F8, are able to relieve CD45 of its GD2-mediated inhibition (lane 2).

Figure 7 shows data from soft-agar clonogenic assays. EL4 GD2^{positive} and EL4 GD2^{negative} cells were cultured in soft agar in the presence or absence of anti-GD2 antibody 3F8 (2 plates per sample).

(a) Total number of visible colonies. EL4 GD2^{positive} cells show reduced colony formation compared to EL4 GD2^{negative} cells. Anti-GD2 antibody 3F8 abolishes EL4 GD2^{positive} cells growth but does not affect EL4 GD2^{negative} cells.

(b) Typical EL4 GD2^{positive} and EL4 GD2^{negative} colonies. EL4 GD2^{negative} colonies contain many more cells than EL4 GD2^{positive} colonies. Bar = 100 micrometers.

Figure 8 shows data from tumorigenic assays. EL4 GD2^{positive} and EL4 GD2^{negative} cells were injected intraperitoneally in a) nude Balb/c and b) syngeneic C57BL/g mice. EL4 GD2^{negative} injected animals (left) show important ascitic tumors, whereas EL4 GD2^{positive} injected animals (right) show localized solid tumors (circled area). Bar = 1 mm.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the invention provides selective artificial ligands of GD2. In some embodiments, such ligands may be used to modulate ganglioside GD2 signal transduction selectively in the tissue where it is normally expressed, particularly in pathways that can be linked to tumorigenic growth or to nociceptive receptors. One aspect of the invention involves the identification of primary and tertiary structures in Tenascin-R that are critical for GD2 binding. In an alternative aspect, the invention demonstrates that a complementarity determining region of anti-GD2 mAb 3F8 is an analogue of Tenascin-R. Small peptide mimics of Tenascin-R and mimics of anti-GD2 mAb 3F8 have accordingly been designed and synthesized as selective ligands of GD2. Alternative ligands may be provided, based on the ligands of the invention, by protein mimicry and antibody mimicry techniques (Saragovi et al., 1992).

In an alternative aspect of the invention, it is demonstrated that GD2 (but not GM1) associates physically with $p56^{Lck}$ *in vivo*, in complexes that are stable to detergent lysis. It is shown that GD2 regulates signal transduction by enhancing $p56^{Lck}$ enzymatic activity *in vitro* and by enhancing *in vivo* phosphorylation of $p56^{Lck}$ and the $p56^{Lck}$ substrate Zap70. Accordingly, GD2 ligands of the invention may be used to cause $p56^{Lck}$-dependent fluxes in intracellular calcium in live cells, and thereby to modulate a variety of physiological or pathological cellular functions.

In an alternative aspect of the invention, it is demonstrated that GD2 (but not other gangliosides) can inhibit CD45 phosphatase activity *in vitro* and *ex vivo*. Accordingly, GD2 ligands of the invention may be used to antagonize the inhibitory action of GD2 on CD45 in live cells, and thereby to modulate a variety of physiological, immunological or pathological cellular functions.

In one aspect of the invention, expression of GD2 is shown to alter tumorigenicity *in vitro* and *in vivo*. As shown in the Examples, in EL4 clones that are otherwise phenotypically indistinguishable $GD2^{negative}$ than in $GD2^{positive}$ cells have equal doubling times, but soft agar colony growth is significantly more efficient in $GD2^{negative}$ cells. Further, addition of GD2 ligands in this assay causes the apoptotic death of $GD2^{positive}$ cells. $GD2^{negative}$ cells are also more tumorigenic *in vivo*. $GD2^{positive}$ cells injected intraperitoneally or subcutaneously in syngeneic mice only form solid tumors at the primary site, with little metastasis. In contrast, $GD2^{negative}$ cells can digest the peritoneal membrane, form viscous, muscin-like ascitic tumors, small solid tumor nodules, and are highly metastatic. Accordingly, in one aspect of the invention cells may be transformed so that they are $GD2^{positive}$, and the transformed $GD2^{positive}$ cells may be treated to GD2 ligands of the invention to mediate cell death, for example by an apoptotic pathway. For example, $GD2^{negative}$ cancer cells may be transformed by targeted gene therapy techniques, so that the cells become $GD2^{positive}$, and the transformed $GD2^{positive}$ cancer cells may be treated with a GD2 ligand of the invention.

In alternative aspects, GD2 ligands of the invention may be used to influence or modulate signal transduction in a biologically relevant manner *in vitro* and *in vivo* in the treatment of diseases such as cancer and in managing symptoms such as pain .

In one aspect, the invention provides compounds, such as GD2 ligands, that are purified, isolated or substantially pure. A compound is "substantially pure" when it is separated from the components that naturally accompany it. Typically, a compound is substantially pure when it is at least 60%, more generally 75% or over 90%, by weight, of the total material in a sample. Thus, for example, a polypeptide that is chemically synthesised or produced by recombinant technology will generally be substantially free from its naturally associated components. A nucleic acid molecule is substantially pure when it is not immediately contiguous with (i.e., covalently linked to) the coding sequences with which it is normally contiguous in the naturally occurring genome of the organism from which the DNA of the invention is derived. A substantially pure compound can be obtained, for example, by extraction from a natural source; by expression of a recombinant nucleic acid molecule encoding a polypeptide compound; or by chemical synthesis. Purity can be measured using any appropriate method such as column chromatography, gel electrophoresis, HPLC, etc.

The term "alkyl" refers to the radical of saturated aliphatic groups, including straight chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. Typical alkyl groups include, but are not limited to, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, *t*-butyl, pentyl, isopentyl, hexyl, etc. The alkyl groups can be (C_1-C_6) alkyl, or (C_1-C_3) alkyl. A "substituted alkyl" has substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, halogen, hydroxyl, carbonyl (such as carboxyl, ketones (including alkylcarbonyl and arylcarbonyl groups), and esters (including alkyloxycarbonyl and aryloxycarbonyl groups)), thiocarbonyl, acyloxy, alkoxyl, phosphoryl, phosphonate, phosphinate, amino, acylamino, amido, amidine, imino,

cyano, nitro, azido, sulfhydryl, alkylthio, sulfate, sulfonate, sulfamoyl, sulfonamido, heterocyclyl, aralkyl, or an aromatic or heteroaromatic moiety. The moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of aminos, azidos, iminos, amidos, phosphoryls (including phosphonates and phosphinates), sulfonyls (including sulfates, sulfonamidos, sulfamoyls and sulfonates), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), $-CF_3$, -CN and the like. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxys, alkylthios, aminoalkyls, carbonyl-substituted alkyls, $-CF_3$, -CN, and the like.

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively. An "alkenyl" is an unsaturated branched, straight chain, or cyclic hydrocarbon radical with at least one carbon-carbon double bond. The radical can be in either the *cis* or *trans* conformation about the double bond(s). Typical alkenyl groups include, but are not limited to, ethenyl, propenyl, isopropenyl, butenyl, isobutenyl, *tert*-butenyl, pentenyl, hexenyl, etc. An "alkynyl" is an unsaturated branched, straight chain, or cyclic hydrocarbon radical with at least one carbon-carbon triple bond. Typical alkynyl groups include, but are not limited to, ethynyl, propynyl, butynyl, isobutynyl, pentynyl, hexynyl, etc.

A "substantially identical" sequence is an amino acid or nucleotide sequence that differs from a reference sequence only by one or more conservative substitutions, as discussed herein, or by one or more non-conservative substitutions, deletion, or insertions located at positions of the sequence that do not destroy the biological function of the test compound. Such a sequence can be at least 60% or 75%, or more generally at least 80%, 85%, 90%, or 95%, or as much as 99% identical at the amino acid or nucleotide level to the sequence used for comparison. Sequence identity can be readily measured using publicly available sequence analysis software (e.g., Sequence Analysis Software Package of the

Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, or BLAST software available from the National Library of Medicine). Examples of useful software include the programs, Pile-up and PrettyBox. Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications.

In alternative embodiments, GD2 ligands can be produced by substitution of either/or (i) side chains, (ii) backbone, or (iii) ionic interaction within a peptide. Additionally, structural or functional analogs can include 1) homologs of the peptidic GD2 ligands generated by peptidomimicry and 2) analogs where the sequence/structure of the GD2 ligands is introduced in a larger protein to convey GD2 binding to that protein.

In one aspect the invention provides nucleic acids that encode peptide compounds of the invention. Such nucleic acids may be introduced into cells for expression using standard recombinant techniques for stable or transient expression. Nucleic acid molecules of the invention may include any chain of two or more nucleotides including naturally occurring or non-naturally occurring nucleotides or nucleotide analogues.

Various genes and nucleic acid sequences of the invention may be recombinant sequences. The term "recombinant" means that something has been recombined, so that when made in reference to a nucleic acid construct the term refers to a molecule that is comprised of nucleic acid sequences that are joined together or produced by means of molecular biological techniques. The term "recombinant" when made in reference to a protein or a polypeptide refers to a protein or polypeptide molecule which is expressed using a recombinant nucleic acid construct created by means of molecular biological techniques. The term "recombinant" when made in reference to genetic composition refers to a gamete or progeny or cell or genome with new combinations of alleles that did not occur in the parental genomes. Recombinant nucleic acid constructs may include a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid

sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Referring to a nucleic acid construct as 'recombinant' therefore indicates that the nucleic acid molecule has been manipulated using genetic engineering, i.e. by human intervention. Recombinant nucleic acid constructs may for example be introduced into a host cell by transformation. Such recombinant nucleic acid constructs may include sequences derived from the same host cell species or from different host cell species, which have been isolated and reintroduced into cells of the host species. Recombinant nucleic acid construct sequences may become integrated into a host cell genome, either as a result of the original transformation of the host cells, or as the result of subsequent recombination and/or repair events.

In one aspect the invention provides antibodies that recognize compounds of the invention, and anti-idiotypic antibodies that in turn recognize such antibodies. The compounds of the invention can be used to prepare antibodies to GD2 ligands using standard techniques of preparation as, for example, described in Harlow and Lane (Antibodies; A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988), or known to those skilled in the art. Antibodies can be tailored to minimise adverse host immune response by, for example, using chimeric antibodies contain an antigen binding domain from one species and the Fc portion from another species, or by using antibodies made from hybridomas of the appropriate species.

Compounds of the invention can be prepared, for example, by replacing, deleting, or inserting an amino acid residue of a GD2 ligand or domain of the invention, with other conservatived amino acid residues, i.e., residues having similar physical, biological, or chemical properties, and screening for biological function. It is well known in the art that some modifications and changes can be made in the structure of a polypeptide without substantially altering the biological function of that peptide, to obtain a biologically equivalent polypeptide. The peptides, ligands and domains of the present invention also extend to biologically equivalent peptides, ligands and domains that differ from a portion of the sequence

of novel ligands of the present invention by conservative amino acid substitutions. As used herein, the term "conserved amino acid substitutions" refers to the substitution of one amino acid for another at a given location in the peptide, where the substitution can be made without substantial loss of the relevant function. In making such changes, substitutions of like amino acid residues can be made on the basis of relative similarity of side-chain substituents, for example, their size, charge, hydrophobicity, hydrophilicity, and the like, and such substitutions may be assayed for their effect on the function of the peptide by routine testing.

In some embodiments, conserved amino acid substitutions may be made where an amino acid residue is substituted for another having a similar hydrophilicity value (e.g., within a value of plus or minus 2.0), where the following may be an amino acid having a hydropathic index of about -1.6 such as Tyr (-1.3) or Pro (-1.6)s are assigned to amino acid residues (as detailed in United States Patent No. 4,554,101, incorporated herein by reference): Arg (+3.0); Lys (+3.0); Asp (+3.0); Glu (+3.0); Ser (+0.3); Asn (+0.2); Gln (+0.2); Gly (0); Pro (-0.5); Thr (-0.4); Ala (-0.5); His (-0.5); Cys (-1.0); Met (-1.3); Val (-1.5); Leu (-1.8); Ile (-1.8); Tyr (-2.3); Phe (-2.5); and Trp (-3.4).

In alternative embodiments, conserved amino acid substitutions may be made where an amino acid residue is substituted for another having a similar hydropathic index (e.g., within a value of plus or minus 2.0). In such embodiments, each amino acid residue may be assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics, as follows: Ile (+4.5); Val (+4.2); Leu (+3.8); Phe (+2.8); Cys (+2.5); Met (+1.9); Ala (+1.8); Gly (-0.4); Thr (-0.7); Ser (-0.8); Trp (-0.9); Tyr (-1.3); Pro (-1.6); His (-3.2); Glu (-3.5); Gln (-3.5); Asp (-3.5); Asn (-3.5); Lys (-3.9); and Arg (-4.5).

In alternative embodiments, conserved amino acid substitutions may be made where an amino acid residue is substituted for another in the same class, where the amino acids are divided into non-polar, acidic, basic and neutral classes,

as follows: non-polar: Ala, Val, Leu, Ile, Phe, Trp, Pro, Met; acidic: Asp, Glu; basic: Lys, Arg, His; neutral: Gly, Ser, Thr, Cys, Asn, Gln, Tyr.

Conservative amino acid changes can include the substitution of an L-amino acid by the corresponding D-amino acid, by a conservative D-amino acid, or by a naturally-occurring, non-genetically encoded form of amino acid, as well as a conservative substitution of an L-amino acid. Naturally-occurring non-genetically encoded amino acids include beta-alanine, 3-amino-propionic acid, 2,3-diamino propionic acid, alpha-aminoisobutyric acid, 4-amino-butyric acid, N-methylglycine (sarcosine), hydroxyproline, ornithine, citrulline, t-butylalanine, t-butylglycine, N-methylisoleucine, phenylglycine, cyclohexylalanine, norleucine, norvaline, 2-napthylalanine, pyridylalanine, 3-benzothienyl alanine, 4-chlorophenylalanine, 2-fluorophenylalanine, 3-fluorophenylalanine, 4-fluorophenylalanine, penicillamine, 1,2,3,4-tetrahydro-isoquinoline-3-carboxylix acid, beta-2-thienylalanine, methionine sulfoxide, homoarginine, N-acetyl lysine, 2-amino butyric acid, 2-amino butyric acid, 2,4,-diamino butyric acid, p-aminophenylalanine, N-methylvaline, homocysteine, homoserine, cysteic acid, epsilon-amino hexanoic acid, delta-amino valeric acid, or 2,3-diaminobutyric acid.

In alternative embodiments, conservative amino acid changes include changes based on considerations of hydrophilicity or hydrophobicity, size or volume, or charge. Amino acids can be generally characterized as hydrophobic or hydrophilic, depending primarily on the properties of the amino acid side chain. A hydrophobic amino acid exhibits a hydrophobicity of greater than zero, and a hydrophilic amino acid exhibits a hydrophilicity of less than zero, based on the normalized consensus hydrophobicity scale of Eisenberg *et al.* (*J. Mol. Bio.* 179:125-142, 1984). Genetically encoded hydrophobic amino acids include Gly, Ala, Phe, Val, Leu, Ile, Pro, Met and Trp, and genetically encoded hydrophilic amino acids include Thr, His, Glu, Gln, Asp, Arg, Ser, and Lys. Non-genetically encoded hydrophobic amino acids include t-butylalanine, while non-genetically encoded hydrophilic amino acids include citrulline and homocysteine.

Hydrophobic or hydrophilic amino acids can be further subdivided based on the characteristics of their side chains. For example, an aromatic amino acid is a hydrophobic amino acid with a side chain containing at least one aromatic or heteroaromatic ring, which may contain one or more substituents such as –OH, -SH, -CN, -F, -Cl, -Br, -I, -NO_2, -NO, -NH_2, -NHR, -NRR, -C(O)R, -C(O)OH, -C(O)OR, -C(O)NH_2, -C(O)NHR, -C(O)NRR, etc., where R is independently (C_1-C_6) alkyl, substituted (C_1-C_6) alkyl, (C_1-C_6) alkenyl, substituted (C_1-C_6) alkenyl, (C_1-C_6) alkynyl, substituted (C_1-C_6) alkynyl, (C_5-C_{20}) aryl, substituted (C_5-C_{20}) aryl, (C_6-C_{26}) alkaryl, substituted (C_6-C_{26}) alkaryl, 5-20 membered heteroaryl, substituted 5-20 membered heteroaryl, 6-26 membered alkheteroaryl or substituted 6-26 membered alkheteroaryl. Genetically encoded aromatic amino acids include Phe, Tyr, and Tryp, while non-genetically encoded aromatic amino acids include phenylglycine, 2-napthylalanine, beta-2-thienylalanine, 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid, 4-chlorophenylalanine, 2-fluorophenylalanine3-fluorophenylalanine, and 4-fluorophenylalanine.

An apolar amino acid is a hydrophobic amino acid with a side chain that is uncharged at physiological pH and which has bonds in which a pair of electrons shared in common by two atoms is generally held equally by each of the two atoms (i.e., the side chain is not polar). Genetically encoded apolar amino acids include Gly, Leu, Val, Ile, Ala, and Met, while non-genetically encoded apolar amino acids include cyclohexylalanine. Apolar amino acids can be further subdivided to include aliphatic amino acids, which is a hydrophobic amino acid having an aliphatic hydrocarbon side chain. Genetically encoded aliphatic amino acids include Ala, Leu, Val, and Ile, while non-genetically encoded aliphatic amino acids include norleucine.

A polar amino acid is a hydrophilic amino acid with a side chain that is uncharged at physiological pH, but which has one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Genetically encoded polar amino acids include Ser, Thr, Asn, and Gln,

while non-genetically encoded polar amino acids include citrulline, N-acetyl lysine, and methionine sulfoxide.

An acidic amino acid is a hydrophilic amino acid with a side chain pKa value of less than 7. Acidic amino acids typically have negatively charged side chains at physiological pH due to loss of a hydrogen ion. Genetically encoded acidic amino acids include Asp and Glu. A basic amino acid is a hydrophilic amino acid with a side chain pKa value of greater than 7. Basic amino acids typically have positively charged side chains at physiological pH due to association with hydronium ion. Genetically encoded basic amino acids include Arg, Lys, and His, while non-genetically encoded basic amino acids include the non-cyclic amino acids ornithine, 2,3,-diaminopropionic acid, 2,4-diaminobutyric acid, and homoarginine.

The above classifications are not absolute and an amino acid may be classified in more than one category. In addition, amino acids can be classified based on known behaviour and or characteristic chemical, physical, or biological properties based on specified assays or as compared with previously identified amino acids. Amino acids can also include bifunctional moieties having amino acid-like side chains.

Conservative changes can also include the substitution of a chemically derivatised moiety for a non-derivatised residue, by for example, reaction of a functional side group of an amino acid. Thus, these substitutions can include compounds whose free amino groups have been derivatised to amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Similarly, free carboxyl groups can be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides, and side chains can be derivatized to form O-acyl or O-alkyl derivatives for free hydroxyl groups or N-im-benzylhistidine for the imidazole nitrogen of histidine. Peptide analogues also include amino acids that have been chemically altered, for example, by methylation, by amidation of the C-terminal amino acid by an alkylamine such as ethylamine, ethanolamine, or ethylene diamine, or acylation

or methylation of an amino acid side chain (such as acylation of the epsilon amino group of lysine). Peptide analogues can also include replacement of the amide linkage in the peptide with a substituted amide (for example, groups of the formula –C(O)-NR, where R is (C_1-C_6) alkyl, (C_1-C_6) alkenyl, (C_1-C_6) alkynyl, substituted (C_1-C_6) alkyl, substituted (C_1-C_6) alkenyl, or substituted (C_1-C_6) alkynyl) or isostere of an amide linkage (for example, -CH_2NH-, -CH_2S, -CH_2CH_2-, -CH=CH- (cis and trans), -C(O)CH_2-, -CH(OH)CH_2-, or –CH_2SO-).

The GD2 ligands, peptides and domains of the invention may be covalently linked, for example, by polymerisation or conjugation, to form homopolymers or heteropolymers. Spacers and linkers, typically composed of small neutral molecules, such as amino acids that are uncharged under physiological conditions, can be used. Linkages can be achieved in a number of ways. For example, cysteine residues can be added at the peptide termini, and multiple peptides can be covalently bonded by controlled oxidation. Alternatively, heterobifunctional agents, such as disulfide/amide forming agents or thioether/amide forming agents can be used. The compound can also be linked to a lipid-containing molecule or peptide that can enhance a T cell response. The compound can also be constrained, for example, by having cyclic portions.

Peptides or peptide analogues can be synthesised by standard chemical techniques, for example, by automated synthesis using solution or solid phase synthesis methodology. Automated peptide synthesisers are commercially available and use techniques well known in the art. Peptides and peptide analogues can also be prepared using recombinant DNA technology using standard methods.

Compounds of the invention can be provided alone or in combination or conjugation with other compounds (for example, toxins, growth factors, anti-apoptotic agents, small molecules, peptides, or peptide analogues), in the presence of a liposome, an adjuvant, or any pharmaceutically acceptable carrier, in a form suitable for administration to humans. Conventional pharmaceutical practice may

be employed to provide suitable formulations or compositions to administer the compounds to patients suffering from diseases such as cancer. Any appropriate route of administration may be employed, for example, parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

If desired, treatment with a compound according to the invention may be combined with more traditional therapies for the disease such as, for example, surgery or chemotherapy.

For therapeutic or prophylactic compositions, the compounds may be administered to an individual in an amount sufficient to induce the destruction of cells (such as cancer cells) or to stop or slow the destruction of cells (such as in neuroprotective treatments or treatment of pain). Amounts considered sufficient will vary according to the specific compound used, the mode of administration, the stage and severity of the disease, the age, sex, and health of the individual being

treated, and concurrent treatments. As a general rule, however, dosages can range from about 1microgram to about 100 mg per kg body weight of a patient for an initial dosage, with subsequent adjustments depending on the patient's response.

In the case of vaccine formulations, an immunogenically effective amount of a compound of the invention can be provided, alone or in combination with other compounds, with an adjuvant, for example, Freund's incomplete adjuvant or aluminum hydroxide. The compound may also be linked with a carrier molecule, such as bovine serum albumin or keyhole limpet hemocyanin to enhance immunogenicity.

In general, compounds of the invention should be used without causing substantial toxicity. Toxicity of the compounds of the invention can be determined using standard techniques, for example, by testing in cell cultures or experimental animals and determining the therapeutic index, i.e., the ratio between the LD50 (the dose lethal to 50% of the population) and the LD100 (the dose lethal to 100% of the population). In some circumstances however, such as in severe disease conditions, it may be necessary to administer substantial excesses of the compositions.

The following examples are intended to illustrate various embodiments and aspects of the invention, and do not limit the invention in any way.

Examples

Methods

Cells: EL4 murine lymphoma and R1.1 murine lymphoma were grown in RPMI 1640 medium (Life Technologies) supplemented with 5% fetal bovine serum, 2 mM glutamine, 10 mM Hepes and penicillin/streptomycin at 37°C in 5% CO_2 humidified atmosphere. A GD2-negative mutant of EL4 was obtained after subcloning of EL4 cells resistant to culture with anti-GD2 mAb 3F8 and rabbit complement.

Flow cytometry: 10^5 cells in 50 µl FACS buffer (PBS, 0.5% BSA, 0.05% NaN_3) were stained for 30 minutes on ice with the following ligands: for GD2, fluorescein isothiocyanate (FITC)-conjugated anti-GD2 mAb 3F8; for GM1, FITC-conjugated cholera toxin B subunit (Sigma); for GM2, rabbit anti-GM2 mAb NANA (Matreya) followed by FITC-conjugated anti-rabbit antibody (Sigma); for GD3, mouse anti-GD3 mAb (Pharmingen) followed by FITC conjugated anti-mouse antibody (Sigma). Cells were washed twice with FACS buffer and analyzed on a flow cytometer (Becton-Dickinson) using CellQuest software.

Detection of GD2 or GM1 by ELISA: 12.5 ng/well of ganglioside (GD2, Advanced Immunochemicals or GM1) were immobilized by drying onto PVC 96-well plates (Falcon), followed by blocking with PBS-0.5% BSA for one hour. Then, anti-GD2 mAb 3F8 or biotin-CTB were added for 10 minutes. The plate was washed three times with PBS-0.5% BSA and incubated 60 minutes with horseradish peroxidase (HRP)-conjugated anti-mouse antibody or HRP-conjugated-avidin. After three washes with PBS-0.5% BSA and two with PBS, the colorimetric substrate ABTS was added and the plate read at 414 nm on a Biorad 550 plate reader.

Co-immunoprecipitation of GD2 and $p56^{Lck}$: cells (5 x 10^6 per sample) were washed in PBS, resuspended in 1 ml lysis buffer (150 mM NaCl, 10 mM sodium phosphate pH 7.2, 2 mM EDTA, 50 mM NaF, 1% CHAPS, 200 µM sodium orthovanadate, 1 mM PMSF, 100 µM leupeptin, 1 mM benzamidine, 300 nM aprotinin, 500 nM soybean trypsin inhibitor) and incubated 30 minutes at 4°C.

Supernatants were collected from lysates after centrifugation (16000 g for 15 minutes at 4°C). Immunoprecipitations were carried out overnight at 4°C using 50 µl protein-G sepharose (Sigma) with either 5 µg of anti-GD2 mAb 3F8 or anti-$p56^{Lck}$ mAb 3A5 (Santa Cruz Biotechnology) or control IgG; or with 50 µl avidin-agarose (Sigma) and 5 µg of biotin-CTB which is specific for GM1 gangliosides. Immunoprecipitates were washed 5 times with 1 ml of cold lysis buffer containing decreasing concentration of CHAPS detergent and samples were extracted with reduced Laemmli buffer. Samples were then used for western blot analysis (see below), or lipids from the samples were isolated for ganglioside quantification by ELISA (see above).

Biochemical analysis: *Western blotting*: the immunoprecipitated samples were fractionated in SDS-PAGE and transferred to membranes, $p56^{Lck}$ immunoblotting was done using rabbit polyclonal anti-$p56^{Lck}$ antibody. *Tyrosine phosphorylation of $p56^{Lck}$ and Zap70*: cells (5 x 10^6 per sample) were washed with PBS, resuspended in 5 ml protein-free hybridoma medium containing 0.2% BSA and allowed to rest for 60 minutes at 37°C to lower baseline kinase activity. Then, 10 µg Anti-GD2 mAb 3F8 (13 nM concentration) or non-specific mouse IgG were added for 5 and 20 minutes. Samples were then washed in cold PBS, detergent solubilized in lysis buffer and immunoprecipitated using 5 µg of anti-$p56^{Lck}$ mAb 3A5 (Santa Cruz Biotechnology) or anti-Zap70 mAb LR (Santa Cruz Biotechnology). Samples were then analyzed by western blotting using anti-phosphotyrosine mAb 4G10. *In vitro $p56^{Lck}$ kinase activity*: $p56^{Lck}$ immunoprecipitates were incubated for 20 minutes at 37°C with $p56^{Lck}$-specific substrates GAP p62 or Sam68 in a kinase reaction buffer (10 mM $MnCl_2$, 20 mM Tris-HCl pH 7.4, 2.5 µM ATP, 20 µCi [γ-^{32}P]ATP). The reaction was stopped by addition of reduced Laemmli buffer and boiling. Phosphorylation of $p56^{Lck}$ substrates was visualized and quantified by SDS-PAGE followed by analysis on a Storm 840 phosphorimager with ImageQuant software.

Effect of exogenous GD2 on $p56^{Lck}$ *in vitro* kinase assay: chromatographically purified $p56^{Lck}$ tyrosine kinase (Upstate Biotechnology) was

incubated on ice for 10 minutes in kinase buffer (100 mM Tris-HCl, pH 7.2, 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2mM EGTA, 150 μM ATP, 0.25 mM sodium orthovanadate, 2 mM DTT) with or without ~20 fold molar excess of ganglioside and 10 μCi [γ-^{32}P]ATP. Then 10 μg of the Src kinase substrate peptide p34^{cdc2}(6-20) (Upstate Biothechnology) was added to the mix and incubated at room temperature for the times indicated, after which the reaction was stopped by addition of 100 μM iodoacetamide and by precipitation of proteins with final 10% TCA. The supernatant (containing the phosphorylated peptide substrate, not precipitated by TCA) was spotted on a P81 paper (Whatman) and washed three times with 0.75% phosphoric acid and once with acetone. The [^{32}P]-peptide content was quantified using a Storm 840 phosphorimager with ImageQuant software.

GD2 ligand design and synthesis: Peptides were modeled based on Tenascin-R sequence and on diversity within Tenascin family homologs. Peptides were synthesized with an Advanced Chemtech automatic synthesizer using solid-phase Fmoc chemistry. After cleavage from resin and side-chain deprotection, peptides containing terminal cysteines were subjected to cyclization by oxydation at 4°C under O_2 in 50 mM ammonium bicarbonate, pH 8.5. Peptides were purified (>95%) by HPLC (Varian) using a C-18 preparative column (Phenomenex), and were verified by Mass Spectometry.

Assessment of peptide-GD2 interaction by competitive ELISA: ELISAs were as described above, except that peptides (50 μg/well, in PBS) were added to the wells for 1 hour before anti-GD2 mAb 3F8 or anti-GM1 cholera toxin-B. Selectivity of inhibition is controlled by lack of effect upon GM1-CTB interactions.

Intracellular calcium studies: cells (1 x 10^6 per sample) were washed with Ringer's solution (155 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 5 mM HEPES) and resuspended in 1 ml Ringer's containing 10 μM p56^{Lck} inhibitor PP1 (BioMol) or vehicle for 15 minutes at 37°C. Then 5 μM Rhod-2AM (Molecular Probes) was then added to the cells and incubated for 30 minutes at room temprature with mild agitation. Cells were washed once in Ringer's,

resuspended in 1 ml Ringer's and incubated for 30 minutes, after which cells were stimulated with the indicated ganglioside ligands (or calcium ionophore A23187 as control) and analyzed over a 30 minute period for intracellular Ca^{2+} using a flow cytometer (Becton-Dickinson).

Soft agar colony formation: single cell suspensions of EL4 $GD2^{negative}$ and EL4 $GD2^{positive}$ were plated (1500 cells/plate) with or without mAb 3F8 (50 µg/plate) in top layer medium (RPMI 1640 containing 0.35% agar, 15% fetal bovine serum, 2 mM glutamine, 10 mM Hepes and penicillin/streptomycin) on top of a preformed bottom layer (same as above except 0.5% agar) in 100 mm polystyrene dishes (Falcon) and grown until colonies are visible by eye. Colonies were counted in a predetermined area of the plates.

***In vivo* tumor studies:** single cell suspensions of EL4 $GD2^{negative}$ and EL4 $GD2^{positive}$ in Hanks' balanced salt solution (Gibco) were injected intraperitonially in nude Balb/c mice (Charles River) (1×10^6 cells per animal). Animals were sacrificed and autopsied after 7 to 10 days, depending on tumor growth.

***In vitro* CD45 phosphatase assay:** human recombinant CD45 enzyme (BioMol, 75 units/well) was preincubated in the absence (negative control) or presence of sodium orthovanadate (positive control) or various gangliosides (16.7 µM) for 20 minutes at room temperature in half-volume 96-well microtiter plates. A CD45-specific substrate ($pp60^{Src}$ C-terminal phosphoregulatory peptide, 200 µM) was then added for 20 minutes at 30°C, afterwhich the reaction was stopped and quantified with BioMol Green reagent. Plates were read at 620 nm with a Biorad 550 platew reader.

***Ex vivo* CD45 assay:** single cell suspensions of EL4 $GD2^{negative}$ and EL4 $GD2^{positive}$ (1×10^7 cells/ml per treatment) were incubated in the presence of 5 µg/ml of mIg (negative control), anti-GD2 antibody 3F8 or anti-CD3 antibody 145-2C11 (Pharmingen) cross-linked with anti-hamster G94-56 (Pharmingen) for 20 minutes at 37°C in RPMI 1640 medium supplemented with 5% fetal bovine serum,

2 mM glutamine, 10 mM Hepes and penicillin/streptomycin. Cells were then washed and detergent lysed. $p56^{Lck}$ was then immunoprecipitated with anti-$p56^{Lck}$ antibody coated beads and probed for phosphotyrosine 505 by western blotting with anti-PY505 (Cell Signaling Technology).

Results

To study a specific physical association between $p56^{Lck}$ and gangliosides, a panel of cells was generated varying in expression of cell surface GD2, GM1, GM2 and GD3, as assessed by flow cytometry. The indicated cells (Table 2) express similar levels of $p56^{Lck}$ (data not shown).

Table 2 Surface ganglioside expression of cells studied as assessed by FACScan

Cells	Source	Ganglioside profile				$p56^{Lck}$ expression
		GM1	GD2	GD3	GM2	
EL4 $GD2^{positive}$	lymphoma mouse C57/bl6n	Low	high	negative	negative	High
EL4 $GD2^{negative}$	lymphoma mouse C57/bl6n	Low	negative	medium	negative	High
R1.1	lymphoma mouse C58/J	High	low	negative	negative	High

Specific and stable association of $p56^{Lck}$–GD2.

We performed anti-GD2 immunoprecipitations with mAb 3F8 followed by western blotting with anti-$p56^{Lck}$ antibodies (Figure 2A). In EL4 $GD2^{positive}$ cells $p56^{Lck}$ was co-precipitated with mAb 3F8 but not with control mouse IgG. In control R1.1 cells, which are $GD2^{negative}$, no antibody co-precipitated $p56^{Lck}$, although $p56^{Lck}$ is expressed at high levels.

The presence and kinase activity of $p56^{Lck}$ in anti-GD2 immunoprecipitates were verified and quantified (Figures 2B and 2C). The indicated immunoprecipitations were done on EL4 $GD2^{positive}$ or EL4 $GD2^{negative}$ cells, followed by *in vitro* kinase assays using the specific $p56^{Lck}$ substrate Sam68. In EL4 $GD2^{positive}$ approximately 15% of the $p56^{Lck}$ activity was co-precipitated by anti-GD2 mAb 3F8 (Figure 2B, lane 1), compared with anti-Lck mAb 3A5 (Figure 2B, lane 2).

In fact, anti-GD2 co-precipitation of the $p56^{Lck}$ activity was comparable to co-precipitation by antibodies against Zap70 (Figure 2B, lane 3). This was in keeping with the fact that Zap70 and $p56^{Lck}$ are reportedly associated physically and functionally *in vivo*.

The specificity of these assays is validated in studies using EL4 $GD2^{negative}$ cells, where anti-GD2 mAb 3F8 did not co-precipitate a $p56^{Lck}$ activity (Figure 2B, lane 4), while anti-Lck and anti-Zap70 antibodies did (Figure 2B, lanes 5 and 6). Because $p56^{Lck}$ co-immunoprecipitations with anti-Zap70 antibodies were comparable in EL4 $GD2^{positive}$ and EL4 $GD2^{negative}$ cell lines (Figure 2B, lanes 3 and 6), the data suggest that association of Zap70 and $p56^{Lck}$ can be GD2 independent.

Similar studies were done using EL4 $GD2^{positive}$ or R1.1 ($GD2^{negative}$) cells and assaying a $p56^{Lck}$ activity by *in vitro* kinase activity upon the selective substrate Gap62 (Figure 2C). Again, anti-GD2 co-purified a $p56^{Lck}$ activity that corresponds to ~15% of the activity purified with anti-Lck mAb 3A5. In contrast, affinity isolation of GM1 with cholera toxin b subunit-coupled beads did not co-purify a $p56^{Lck}$ activity (Figure 2C, lane 4), although R1.1 cells have high levels of GM1 and $p56^{Lck}$ (Figure 2C, lane 3).

The converse experiment was performed where $p56^{Lck}$ was first immunoprecipitated with mAb 3A5 and then the presence of GD2 or GM1 were gauged by ELISA (Figure 2D). Anti-$p56^{Lck}$ immunoprecipitates from EL4 $GD2^{positive}$ cells contained ~55% of the GD2 that could be isolated with anti-GD2 mAb 3F8. In specificity controls, anti-$p56^{Lck}$ immunoprecipitates from R1.1 cells did not co-precipitate the ganglioside GM1.

Functional relevance of $p56^{Lck}$–GD2 association in the absence of GD2 ligands.

Subsequent experiments (Figure 3) addressed *in vitro* whether there is a functional relevance to the stable and selective GD2-$p56^{Lck}$ association. Addition of exogenous GD2 to purified $p56^{Lck}$ increased the kinetics of *in vitro* enzymatic

activity from a $t_{1/2}$ of ~25 minutes to a $t_{1/2}$ of ~17 minutes, with a dramatic 70% increase in kinase activity at 20 minutes. However, the v_{max} did not change, as the enzyme activity reaches a similar plateau with or without GD2 present. In control assays, $p56^{Lck}$ kinase activity was not altered upon addition of exogenous GM1 (Figure 3), or addition of phosphatidylcholine or other lipids (data not shown).

Development of artificial GD2 ligands.

Small peptides (6-13 amino acids in length) were designed to span the primary sequence of the GD2 ligand Tenascin-R. Cyclization and other conformational constrains were introduced in these peptides to conformationally constrain them.and to forcethem to adopt the β-turn structures desired.

These peptides (~100 were synthesized) were tested for inhibition of GD2–mAb 3F8 interactions in ELISA plates (Table 3). No competitor peptide added or control irrelevant peptide added were standardized as 100% binding. For simplicity, only some of the ~90 inactive peptides are shown.

In various embodiments, peptide analogs that actively inhibit GD2-mAb 3F8 interactions span the sequence NH_2-Ile-Thr(Ala)-Asn-Tyr-Asn-COOH. In some embodiments, peptides of the invention may be in type IV (3:5 or 3:3) canonical β-turn configurations with a distance between the Cα1 to Cα4 atom varying between 4 and 7 Å, depending on whether Gly residues (underlined) are incorporated in the sequence. Additionally, a *linear* peptide of 11 amino acids (pep 51), which is unstructured in solution (data not shown) can also act as a competitive inhibitor.

Table 3. Selected peptide mimics..

Peptides	Sequence	Conformation	% Inhibition of mAb 3F8–GD2 interactions
Pep 51	**G G I T N Y N** S A L M	linear	**44.96 ± 1.57**
Pep 52	C **G G I T N Y N S A** C	cyclic	30.17 ± 2.69
Pep 53	C **I T N Y N S** C	cyclic	24.74 ± 3.29
Pep 54	C **G G I T N Y N** C	cyclic	**40.34 ± 3.17**
Pep 55	C **T N Y G V H** C	cyclic	12.23 ± 4.41
Pep 56	C **T N Y G V** C	cyclic	14.13 ± 5.43
Pep 57	**G G I A N Y N T S**	linear	4.33 ± 5.23
Pep 58	C **G G I A N Y N** C	cyclic	**47.60 ± 4.26**
Pep 59	C **G G I A N Y N T S** C	cyclic	**48.83 ± 5.01**
Pep 60	C **I A N Y N T** C	cyclic	30.94 ± 3.53

Inhibition of mAb 3F8 bindng to immobilized GD2 by peptides, measured by competitive ELISA. No peptide or control peptide treatments are standardized as 0% inhibition. No mAb 3F8 treatment is standardized as 100% inhibition. Average of 5 indepedant experiments, n=4 in each experiment. Mean ± SEM.

Functional relevance of GD2 ligands in $p56^{Lck}$ activation.

This example illustrates (i) whether engagement of cell surface GD2 with ligands causes the pTyr of $p56^{Lck}$ (Figure 4A); (ii) whether engagement of cell surface GD2 with ligands leads to the pTyr of the downstream effector Zap70 (Figure 4B); (iii) whether engagement of cell surface GD2 induces intracellular calcium fluxes (Figure 4C).

First, engagement of cell surface GD2 in living EL4 $GD2^{positive}$ cells with the artificial GD2 ligand mAb 3F8 caused the pTyr of $p56^{Lck}$ within 5 min *in vivo* (Figure 4A, lanes 3 and 4). Control mouse IgG did not cause this effect (Figure 4A, lanes 1 and 2).

Second, since the pTyr of $p56^{Lck}$ can lead either to activation or to inactivation of this kinase depending on which Tyr residue gets phosphorylated, the pTyr profile of Zap70 was studied *in vivo* because it is an adapter molecule downstream of and phosphorylated by activated $p56^{Lck}$. Zap70 is transiently pTyr upon engagement of cell-surface GD2 with mAb 3F8 as a ligand (Figure 4B, lanes 3 and 4). This Zap70 pTyr presumably occurs via $p56^{Lck}$. Control mouse IgG did not cause Zap70 pTyr in EL4 $GD2^{positive}$ cells (Figure 4B, lanes 1 and 2). In other specificity controls, treatment of EL4 $GD2^{negative}$ cells with GD2 ligands did not cause pTyr of $p56^{Lck}$ or Zap70 (data not shown) indicating that cell surface GD2 expression may be required for ligand engagement of GD2 and a subsequent effect downstream, in some embodiments.

Third, intracellular Ca^{++} concentrations were measured in live EL4 $GD2^{positive}$ cells after engagement of cell-surface GD2 with ligands (Figure 4C). Flow cytometric analysis using the Ca^{++} sensitive Rhod-2 fluorophore showed a rapid (~5 minutes) 1.6 fold increase of intracellular Ca^{++} after mAb 3F8 binding, but not after binding by control mouse antibody or CTB (data not shown). The selective $p56^{Lck}$ kinase inhibitor PP1 (10 μM) markedly decreased mAb 3F8-induction of Ca^{++} fluxes. This control suggests that activated $p56^{Lck}$ may be responsible for aspects of the GD2 signal transduction pathway leading to intracellular Ca^{++} fluxes in some embodiments.

Functional relevance of the presence of GD2 on CD45 activity.

Since the CD45 phosphatase is responsible for the activation of $p56^{Lck}$, the effect of GD2 on CD45 activity was investigated. First, the effect of GD2 was tested *in vitro* using human recombinant CD45 enzyme and $pp60^{Src}$ phosphoregulatory peptide as a substrate. As seen in figure 5, GD2 can drastically inhibit CD45 phosphatase activity (85% inhibition), while other gangliosides (GM1, GM2, GD3 and GM3) had little influence on the enzymatic activity of CD45. Other than GD2, only GD1a showed significant inhibition (50%).

The inhibitory effect of GD2 on CD45 is also seen in live cells, as shown in figure 6. Here, EL4 GD2^{positive} and EL4 GD2^{negative} cells were stimulated using the well-documented T cell receptor cross-linking methodology, which typically results in activation of p56^{Lck} following its dephosphorylation at tyrosine 505 by CD45. Interestingly, EL4GD2^{positive} cells are resistant to activation (Figure 6, lane 3), while the EL4 GD2^{negative} cells (Figure 7, lane 6) are readily activated upon T cell receptor cross-linking. This discrepancy can be attributed to the presence of GD2 in EL4 GD2^{positive} cells which can block CD45-mediated activation of p56^{Lck}. Of interest is the fact that GD2 ligands, such as mAb 3F8, seem to be able to alleviate GD2 inhibition of CD45 and allow for T cell receptor activation as seen by dephosphorylation of tyrosine 505 on p56^{Lck} (Figure 6, lane 2).

Functional relevance of GD2 and GD2 ligands in tumorigenic growth.

To illustrate the effect of cell surface expression of GD2 on cancer cell growth and survival, EL4 GD2^{positive} and EL4 GD2^{negative} cells were grown in clonoenic (soft agar) assays (Figure 7), or they were injected *in vivo* intraperitoneally (Figure 8).

Colony formation assays in soft agar showed marked differences in growth dynamics for EL4 GD2^{positive} and EL4 GD2^{negative} cells. At day 10 of growth, the total number of EL4 GD2^{positive} colonies per plate were lower by 50% compared to EL4 GD2^{negative} colonies (Figure 7a). Moreover the EL4 GD2^{negative} colonies were much larger and contained more cells (Figure 7b). This is striking because both cell lines have identical doubling times in liquid culture.

Addition of mAb 3F8 in the agar layer (where it putatively diffuses, engages and activates GD2-mediated signals) totally abolished EL4 GD2^{positive} colony formation while having no consequences on the number and size of EL4 GD2^{negative} colonies. This illustrates that the GD2 ligands of the invention may be used to treat GD2 positive cells to modulate growth, for example to treat cancer cells expressing GD2 to inhibit the growth of such cells or to kill the cells. In some embodiments, EL4 GD2^{positive} cells die by apoptosis when GD2 is bound (data not shown),

indicating that the presence of GD2 may in some embodiments allow the GD2 ligands of the invention to be used to induce apoptosis.

An evident difference is also observed when cells are grown *in vivo*. When injected intraperitoneally EL4 $GD2^{negative}$ cells mainly form aggressive and metastatic ascitic tumors and the peritoneal cavity contains mucin-like peptidoglycans. In contrast, EL4 $GD2^{positive}$ cells form localized, highly vascularized solid tumors attached to the peritoneal membrane (Figure 8).

Conclusion

Although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. Numeric ranges are inclusive of the numbers defining the range. In the specification, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to", and the word "comprises" has a corresponding meaning. Citation of references herein shall not be construed as an admission that such references are prior art to the present invention. All publications, including but not limited to patents and patent applications, cited in this specification are incorporated herein by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein and as though fully set forth herein. The invention includes all embodiments and variations substantially as hereinbefore described and with reference to the examples and drawings.

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The following documents are hereby incorporated by reference:

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